EXHIBIT A64

Journal of Toxicology and Environmental Health, Part A, 73:423–436, 2010

ISSN: 1528-7394 print / 1087-2620 online
DOI: 10.1080/15287390903486568

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UTILIZATION OF GENE PROFILING AND PROTEOMICS TO DETERMINE MINERAL PATHOGENICITY IN A HUMAN MESOTHELIAL CELL LINE (LP9/TERT-1)

Jedd M. Hillegass[1], Arti Shukla[1], Maximilian B. MacPherson[1], Jeffrey P. Bond[2], Chad Steele[3], Brooke T. Mossman[1]

[1]Department of Pathology, University of Vermont College of Medicine, Burlington, Vermont
[2]Department of Microbiology and Molecular Genetics, University of Vermont College of Medicine, Burlington, Vermont
[3]Departments of Medicine and Microbiology, University of Alabama at Birmingham School of Medicine, Birmingham, Alabama, USA

Identifying and understanding the early molecular events that underscore mineral pathogenicity using *in vitro* screening tests is imperative, especially given the large number of synthetic and natural fibers and particles being introduced into the environment. The purpose of the work described here was to examine the ability of gene profiling (Affymetrix microarrays) to predict the pathogenicity of various materials in a human mesothelial cell line (LP9/TERT-1) exposed to equal surface area concentrations (15×10^6 or 75×10^6 $\mu m^2/cm^2$) of crocidolite asbestos, nonfibrous talc, fine titanium dioxide (TiO_2), or glass beads for 8 or 24 h. Since crocidolite asbestos caused the greatest number of alterations in gene expression, multiplex analysis (Bio-Plex) of proteins released from LP9/TERT-1 cells exposed to crocidolite asbestos was also assessed to reveal if this approach might also be explored in future assays comparing various mineral types. To verify that LP9/TERT-1 cells were more sensitive than other cell types to asbestos, human ovarian epithelial cells (IOSE) were also utilized in microarray studies. Upon assessing changes in gene expression via microarrays, principal component analysis (PCA) of these data was used to identify patterns of differential gene expression. PCA of microarray data confirmed that LP9/TERT-1 cells were more responsive than IOSE cells to crocidolite asbestos or nonfibrous talc, and that crocidolite asbestos elicited greater responses in both cell types when compared to nonfibrous talc, TiO_2, or glass beads. Bio-Plex analysis demonstrated that asbestos caused an increase in interleukin-13 (IL-13), basic fibroblast growth factor (bFGF), granulocyte colony-stimulating factor (G-CSF), and vascular endothelial growth factor (VEGF). These responses were generally dose-dependent (bFGF and G-CSF only) and tumor necrosis factor (TNF)-α independent (except for G-CSF). Thus, microarray and Bio-Plex analyses are valuable in determining early molecular responses to fibers/particles and may directly contribute to understanding the etiology of diseases caused by them. The number and magnitude of changes in gene expression or "profiles" of secreted proteins may serve as valuable metrics for determining the potential pathogenicity of various mineral types. Hence, alterations in gene expression and cytokine/

This work was supported by EUROTALC and the Industrial Minerals Association of North America. The authors thank the Vermont Cancer Center DNA Analysis Core Facility (supported in part by grant P30CA22435 from the National Cancer Institute) at the University of Vermont (Burlington) for technical assistance with microarray analysis. We also acknowledge Dr. Pamela Vacek (Department of Medical Biostatistics at the University of Vermont, Burlington) for providing direction in performing statistical analysis and Gary Tomiano (Minteg International, Inc./Specialty Minerals, Inc., Easton, PA) for talc characterization. JMH is also supported by training grant T32 ES007122 from the National Institute of Environmental Health Sciences. Among the authors, BTM and AS conceived of the overall research plan. AS and MBM maintained cell cultures, introduced minerals to cells, collected cell medium, prepared RNA, and performed the microarray analysis. JPB carried out PCA on the acquired microarray data. CS conducted the Bio-Plex analysis. JMH interpreted the data, carried out statistical analysis on the Bio-Plex data, and prepared the article. All authors read and approved the final article.

Address correspondence to Jedd M. Hillegass, Department of Pathology, University of Vermont College of Medicine, 89 Beaumont Avenue, Given E203, Burlington, VT 05405-0068, USA. E-mail: jedd.hillegass@uvm.edu

chemokine changes induced by crocidolite asbestos in LP9/TERT-1 cells may be indicative of its increased potential to cause mesothelioma in comparison to the other nonfibrous materials examined.

Given the large number of synthetic and natural fibers and particles being introduced into the environment, both occupationally and through weathering and consumer products (Hansen et al., 2008; Schulte et al., 2008), it seems prudent to implement various *in vitro* screening tests to predict toxicity and the mechanism(s) of action of these fibers or particles to target cells of disease. Asbestos is of particular interest to our laboratory given its association with the development of a number of both nonmalignant (asbestosis, pleural fibrosis) and malignant (bronchogenic carcinoma, pleural and peritoneal mesothelioma) diseases (Manning et al., 2002; Mossman & Gee, 1989). The pathogenicity of various types of asbestos fibers is generally thought to be associated with fiber size/geometry, habit, and surface composition, leading to increased biopersistence and bioreactivity (Mossman & Churg, 1998). For example, the presence of transition metals on the surface of asbestos fibers is a causative factor leading to the production of reactive oxygen species (ROS) through Fenton and Haber–Weiss chemistry (Hansen & Mossman, 1987; Kamp & Weitzman, 1999; Vallyathan et al., 1992). Cellular effects of asbestos exposure include DNA strand breaks and base alterations (Lund & Aust, 1992; Lund et al., 1994), intracellular glutathione depletion (Janssen et al., 1995; Shukla et al., 2004), alterations in cell signaling and activation of transcription factors (Shukla et al., 2008), induction of various pro-inflammatory signals including cytokines and chemokines (Lemaire & Ouellet, 1996; Robledo & Mossman, 1999), and apoptosis followed by compensatory proliferation (Buder-Hoffmann et al., 2008; Shukla et al., 2003a, 2003b). Furthermore, the early molecular events contributing to the etiology of asbestos-related diseases are still poorly understood. Although studies examining the roles of single genes or proteins have contributed to basic knowledge, a need exists for more comprehensive assays capable of measuring multiple parameters simultaneously, especially in light of the complexity of asbestos responses. Gene profiling through microarrays and multiplex (Bio-Plex) analysis of cytokines/chemokines have the potential to fulfill this need.

The objective of the work described here was to examine the utilization of gene profiling and Bio-Plex analysis in human cells to predict the pathogenicity of various mineral types. To do so, we first employed principal component analysis (PCA) of microarray data (Shukla et al., 2008) to compare the response of a contact-inhibited hTERT-immortalized human mesothelial cell line (LP9/TERT-1) (Dickson et al., 2000) to that of a contact-inhibited SV40 Tag-immortalized human ovarian epithelial cell line (IOSE) (Choi et al., 2004) exposed to crocidolite asbestos, nonfibrous talc, fine titanium dioxide (TiO_2), or glass beads. Although not implicated in asbestos-induced diseases (Committee on Asbestos: Selected Health Effects, 2006; Reid et al., 2009), contact-inhibited IOSE cells have been utilized by many laboratories as a cell line representative of "normal" ovarian epithelial cells (Merritt et al., 2008). Crocidolite asbestos was chosen since it is generally regarded as the most pathogenic asbestos type in the causation of human malignant mesothelioma (MM) (Lake & Robinson, 2005; Mossman et al., 1990). In addition to PCA, we implemented Bio-Plex analysis of a panel of chemokines/cytokines induced in LP9/TERT-1 cells following exposure to crocidolite asbestos and/or tumor necrosis factor-alpha (TNF-α), a known primer essential for stimulation of many chemokines/cytokines in human alveolar macrophages (Dostert et al., 2008). Moreover, TNF-α has been associated with transformation of human mesothelial cells (Yang et al., 2008). Bio-Plex analysis provides a powerful tool for determining changes in a multitude of different analytes, allowing a biologically relevant protein "profile" to be generated.

We hypothesized that the number and magnitude of significant responses determined using microarray analysis would be indicative of the overall pathogenicity of the mineral of interest. Any changes in expression of specific genes/proteins might also provide insight into the molecular events contributing to the etiology of fiber/particle-related diseases. We recently reported that in LP9/TERT-1 human mesothelial cells, crocidolite asbestos caused a greater number of gene changes than nonfibrous talc, TiO_2, or glass beads at comparable surface area concentrations (15×10^6 or 75×10^6 $\mu m^2/cm^2$) and times (8, 24 h) (Shukla et al., 2008). In studies here, PCA of these data confirmed both that LP9/TERT-1 cells were more responsive than IOSE cells to crocidolite asbestos or nonfibrous talc, and that exposure to crocidolite asbestos elicited a greater response in either cell type when compared to nonfibrous talc, TiO_2, or glass beads. Further, novel data from Bio-Plex analysis demonstrated that crocidolite asbestos caused an increase in interleukin-13 (IL-13), basic fibroblast growth factor (bFGF), granulocyte colony-stimulating factor (G-CSF), and vascular endothelial growth factor (VEGF). These responses were generally dose dependent (bFGF and G-CSF only) and TNF-α independent (except for G-CSF), suggesting that asbestos may elicit a number of autocrine growth factor pathways in human mesothelial cells. Overall, our data not only afford an indication of the early molecular events following mineral exposure, but also suggest that the number and magnitude of changes elicited by fibers or particles may be indicative of their pathogenicity.

METHODS

Human Mesothelial and Ovarian Epithelial Cell Cultures

Human mesothelial LP9/TERT-1 cells, a contact-inhibited hTERT-immortalized cell line phenotypically and functionally resembling normal human peritoneal mesothelial cells (Dickson et al., 2000), were obtained from Dr. James Rheinwald (Dana Farber Cancer Research Institute, Boston). LP9/TERT-1 cells were maintained in DMEM/F-12 (1:1) medium (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (FBS) (Mediatech), penicillin–streptomycin (50 U/ml penicillin G, 50 µg/ml streptomycin sulfate) (GIBCO, Carlsbad, CA), hydrocortisone (100 µg/ml), insulin (2.5 µg/ml), transferrin (2.5 µg/ml), and selenium (2.5 µg/ml) (Sigma, St. Louis, MO). For microarray studies, human ovarian epithelial IOSE 398 cells, which are SV40 Tag-immortalized and anchorage dependent (Choi et al., 2004), were obtained from Dr. Nelly Auersperg (Canadian Ovarian Tissue Bank, University of British Columbia, Vancouver, BC, Canada). IOSE cells were maintained in 50:50 199/MCB105 medium containing 10% FBS and gentamicin (50 µg/ml). Cells at near confluency were switched to maintenance medium containing 0.5% FBS overnight prior to fiber or particle exposure.

Introduction of Minerals to Cells

The physical and chemical characterization of all fibers and particles used in these studies has been reported previously (Shukla et al., 2008). Nitrogen gas sorption analysis was utilized to measure the surface area of all fibers/particles used in these studies to allow equal-surface-area amounts to be added to cells. The mean surface areas (± SEM) of the fibers/particles of interest were as follows: crocidolite asbestos (14.97 ± 0.605 m^2/g), talc (16.03 ± 0.654 m^2/g), titanium dioxide (9.02 ± 0.185 m^2/g), and glass beads (2.78 ± 0.215 m^2/g). Following sterilization under ultraviolet light overnight, minerals were suspended in 1× Hanks balanced salt solution (HBSS) at 1 mg/ml, sonicated for 15 min in a water-bath sonicator, and triturated 5 times through a 22-gauge needle. This suspension was added to cells in medium to achieve the desired surface area-based concentrations of 15×10^6 and 75×10^6 $\mu m^2/cm^2$ (equivalent to 1 $\mu g/cm^2$ and 5 $\mu g/cm^2$).

RNA Preparation and Microarray

Total RNA was prepared using an RNeasy Plus Mini Kit according to the manufacturer's protocol (Qiagen, Valencia, CA), as published previously (Shukla et al., 2007). Microarrays were performed on samples from three

independent experiments. For each experiment, $n = 3$ dishes were pooled into one sample per treatment group, giving a total of $n = 3$ RNA samples per group. All procedures were performed by the Vermont Cancer Center DNA Analysis Core Facility using a standard Affymetrix protocol as previously described (Sabo-Attwood et al., 2005; Shukla et al., 2007). GeneChip Human Genome U133A 2.0 arrays (Affymetrix, Santa Clara, CA) targeting 18,400 human transcripts were scanned twice (Hewlett-Packard GeneArray Scanner), the images were overlaid, and the average intensities of each probe cell were compiled. Microarray data were analyzed using GeneSifter software (VizX Labs, Seattle, WA).

Principal Component Analysis (PCA)

Microarray data analysis consisted of (1) calculation of a statistic for each probe set in each sample reflecting expression level, (2) calculation of a statistic for each probe set and cell line reflecting the significance of differential expression among sample groups, (3) filtering to obtain a set of probe sets enriched for those that are differentially expressed, and (4) reduction of the dimensionality of the variation in remaining probe set differential expression statistics.

Robust multi-array average (RMA) (Bolstad et al., 2003; Irizarry et al., 2003) expression statistics were calculated for each probe set in each sample using the Affy package (Irizarry et al., 2007) and the R language and environment for statistical computing (R Development Core Team, 2007). Probe sets not called present in at least one sample by the MAS5 algorithm were removed. The p value associated with the null hypothesis of no differential expression among sample groups of a cell line was calculated using Smyth's limma package (Smyth, 2004, 2005) based on a linear model having one parameter for each sample group. Probe sets were retained that were below the 1% p-value quantile for either LP9/TERT-1 or IOSE cells. Principal components and their coefficients were calculated from these differential expression statistics (without centering or scaling) using R.

Bio-Plex Analysis of Cytokine and Growth Factor Concentrations in Cell Medium Following Exposure to Crocidolite Asbestos and/or TNF-α

To quantify cytokine and chemokine levels in cell medium following administration of 15×10^6 or 75×10^6 $\mu m^2/cm^2$ crocidolite asbestos in the presence or absence of 10 ng/ml TNF-α for 24 h, a multiplex suspension protein array was performed using the Bio-Plex protein array system and a human cytokine 27-plex panel (Bio-Rad) on confluent cells in 35 mm diameter cell culture dishes. This method of analysis is based on Luminex technology and simultaneously measures the following proteins: interleukin-1β (IL-1β), IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic fibroblast growth factor (bFGF), eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), IFN-inducible protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1; MCAF), macrophage inflammatory protein-1α (MIP-1α), MIP-1β, platelet-derived growth factor-BB (PDGF-BB), regulated on activation normal T-cell expressed and secreted (RANTES), tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor (VEGF). Briefly, anti-cytokine/chemokine antibody-conjugated beads were added to individual wells of a 96-well filter plate and adhered using vacuum filtration. After washing, 50 µl of prediluted standards (range = 32,000 pg/ml to 1.95 pg/ml) or cell medium ($n = 3$/group) was added, and the filter plate was shaken at 300 rpm for 30 min at room temperature. Thereafter, the filter plate was washed, and 25 µl of prediluted multiplex detection antibody was added for 30 min. After washing, 50 µl of prediluted streptavidin-conjugated phycoerythrin was added for 10 min, followed by an additional wash and the addition of 120 µl of Bio-Plex assay buffer to each well. The filter plate was analyzed using the Bio-Plex protein array system, and concentrations of each cytokine and chemokine were determined using Bio-Plex Manager Version

3.0 software. Data are expressed as pg of cytokine per ml of medium.

Statistical Analysis

Bio-Plex data are expressed as the mean ± standard error of the mean (SEM). Statistical differences between defined groups were evaluated using Student's *t*-test. Differences with p values $<.05$ were considered statistically significant.

RESULTS

Principal Component Analysis (PCA) Suggests the Response of LP9/TERT-1 Cells to Crocidolite Asbestos Is Greater Than That of IOSE Cells

A summary of the total numbers of gene changes (>2-fold in comparison to untreated controls) generated via microarray analysis and previously reported (Shukla et al., 2008) is provided for LP9/TERT-1 cells (Table 1) and IOSE cells (Table 2) following exposure to various fiber/particle preparations. PCA was subsequently used to quantify patterns of differential gene expression. The numbers and identities of genes exceeding 2-fold differential expression (Shukla et al., 2008) suggest three features of the transcriptional response of these two cell lines to fibers/particles: (1) The responses of LP9/TERT-1 cells to the range of minerals, doses, and times investigated differ in quantity, but not substantially in quality, (2) the responses to minerals by LP9/TERT-1 or IOSE cells differ in quality, not simply in quantity, and (3) the responses of the LP9/TERT-1 cells to asbestos are greater than that of IOSE cells.

To clarify these observations, we subtracted from each of the 45 treated samples the average expression intensities of its associated controls and plotted the coefficients of the first two principal components (Figure 1). The LP9/TERT-1 groups lie approximately along a line through the origin, consistent with the exposures eliciting a response of the same type, but perhaps to different extents. The microarray measurements associate each treated sample with a point in a multidimensional space, a point that captures the differential expression of many genes (those selected by the filtration procedures; see Methods section). A sample at the origin is unperturbed by the treatment, while a sample farther from the origin is more perturbed. The principal component analysis served to identify a two-dimensional subspace that captures a large proportion of the variation in the selected genes among samples. Samples along a line through the origin in this subspace differ in the magnitude, but not in the pattern of the perturbation in gene expression. The exposures can then be partially ordered based on the magnitude of the response: Asbestos 15/24 or 75/8 >> Asbestos 15/8 or Talc 75/8 >> All other minerals, where X/Y indicates an exposure at X × 10^6 $\mu m^2/cm^2$ for Y h. In contrast, the response of the IOSE cells is almost perpendicular to that of the LP9/TERT-1 cells, indicating that it reflects differential gene expression unlike that of the LP9/TERT-1 cells, and suggests the following: Asbestos 75/24 >> All other minerals. Quantified by the length of the coefficients of the principal components (Figure 1) or by the length of differential

TABLE 1. Total Number of Gene Expression Changes in LP9/TERT-1 Human Peritoneal Mesothelial Cells (Shukla et al. 2008)

Mineral name	Concentration ($x10^6$ $\mu m^2/cm^2$)	Number of gene changes >2-fold	
		8 h	24 h
Glass beads	75	0	0
TiO_2	15	0	0
Talc (MP 10-52)	15	1	0
	75	30	Not tested
Crocidolite asbestos	15	29	205
	75	236	Not tested

TABLE 2. Total Number of Gene Expression Changes in IOSE Human Ovarian Cells (Shukla et al. 2008)

Mineral name	Concentration ($x10^6$ $\mu m^2/cm^2$)	Number of gene changes >2-fold	
		8 h	24 h
Glass beads	75	0	0
TiO_2	75	0	0
Talc (MP 10-52)	75	0	0
Crocidolite asbestos	75	2	17

expression vectors (data not shown), the response of LP9/TERT-1 cells to 15×10^6 $\mu m^2/cm^2$ asbestos at 24 h (Figure 1; red inverted triangles) was greater in magnitude than the response of IOSE cells to a higher concentration of asbestos (75×10^6 $\mu m^2/cm^2$) for the same duration (Figure 1; blue inverted triangles).

Crocidolite Asbestos Induces an Array of Cytokines and Growth Factors in LP9/TERT-1 Human Mesothelial Cells

Bio-Plex analysis was carried out on medium collected from LP9/TERT-1 cells following exposure to 15×10^6 or 75×10^6 $\mu m^2/cm^2$ crocidolite asbestos in the presence or absence of 10 ng/ml TNF-α for 24 h. Although data for a full panel of 27 cytokines/chemokines were collected, we chose to focus on IL-13, bFGF, G-CSF, and VEGF for the purpose of this article (Figure 2). Exposure to crocidolite asbestos caused an increase in IL-13, bFGF, G-CSF, and VEGF; however, only bFGF and G-CSF increased in a dose-dependent manner. Specifically, for bFGF, a dose of 15×10^6 $\mu m^2/cm^2$ asbestos caused a 7-fold increase in protein and a dose of 75×10^6 $\mu m^2/cm^2$ asbestos caused a 19-fold increase compared to cells in medium alone (Figure 2B). Similar increases were seen in cells co-treated with asbestos and TNF-α, although TNF-α priming did not have a significant effect. For G-CSF, exposure to 15×10^6 $\mu m^2/cm^2$ and 75×10^6 $\mu m^2/cm^2$ asbestos resulted in a 2-fold and 5-fold increase, respectively, over cells in medium alone (although only the 75×10^6 $\mu m^2/cm^2$ dose was statistically significant; $p < .05$) (Figure 2C). In addition, it appears that coexposing cells to asbestos and TNF-α caused a significant ($p < .05$) increase in the amount of G-CSF protein produced by LP9/TERT-1 cells compared to cells that had been exposed to asbestos alone. However, there was no difference between the sum of the individual responses to asbestos alone and that for TNF-α alone. Concentrations of 15×10^6 $\mu m^2/cm^2$ and 75×10^6 $\mu m^2/cm^2$ asbestos alone caused increases in IL-13 (Figure 2A) and VEGF (Figure 2D) protein, although only changes in IL-13 levels were significant ($p < .05$). These responses appeared to be TNF-α independent.

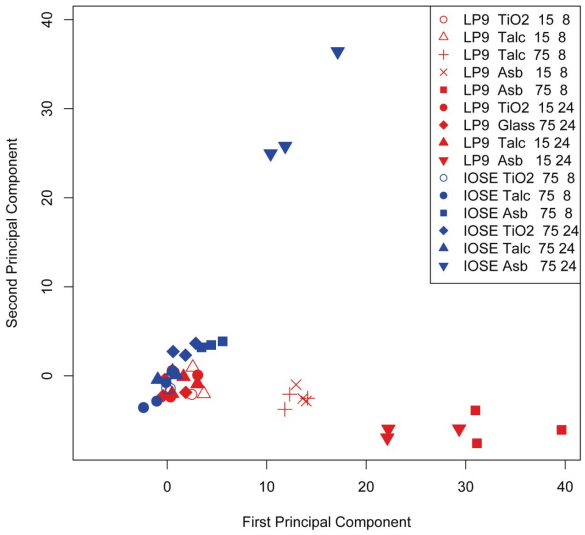

FIGURE 1. Principal component analysis of the response of LP9/TERT-1 and IOSE cells to exposures involving different minerals, doses, or times. A linear LP9/TERT-1 response suggests exposures elicited similar responses, but to different extents, and allows exposures to be partially ordered based on response magnitude as follows: Asbestos 15/24 or 75/8 >> Asbestos 15/8 or Talc 75/8 >> All other minerals ([X × 10^6 $\mu m^2/cm^2$]/Y hours). The fact that the IOSE response is nearly perpendicular to that of LP9/TERT-1 cells suggests differential gene expression, unlike that of LP9/TERT-1 cells, and indicates: Asbestos 75/24 >> All other minerals. Further, if the response is quantified by the length of the coefficients of the principal components or by the length of the differential expression vectors (data not shown): LP9/TERT-1 Asbestos 15/24 >> IOSE Asbestos 75/24.

DISCUSSION

The concept of utilizing gene profiling either to determine the likelihood of a specific pathogenic event or to correlate a gene expression "fingerprint" with a particular mechanism of action is familiar. Not only have models of gene profiling been developed in rodents as a means of classifying the toxicity of compounds to liver and other organs (Steiner et al., 2004), but this concept has previously been applied to define early molecular events following exposure of normal human bronchial epithelial cells (NHBEs) (Belitskaya-Levy et al., 2007), murine nontransformed alveolar type II epithelial (C10) cells (Ramos-Nino et al., 2003), human lung adenocarcinoma (A549) cells (Hevel et al., 2008; Nymark et al., 2007), SV40-transformed

TABLE 3. Summary of Published Gene Profiling Studies Performed on Asbestos-Exposed Cells *In Vitro*

Reference	Mineral	Concentration	Cell Type	Results
(1)	Chrysotile	7 μg/cm^2	NHBE	4 h: 8▲, 0▼ 24 h: 72▲, 3▼
	Summary: Gene profiles regulated by chrysotile asbestos consisted mainly of cytokines, growth factors, and DNA damage genes, although a number of transcription factors were also upregulated. The largest changes in gene expression at 24 h were seen in IL-8 (35-fold increase), CXCL1 (28-fold increase), ATF3 (19-fold increase), and growth arrest and DNA-damage-inducible beta (15-fold increase).			
(2)	Crocidolite	5 μg/cm^2	C10	24 h: 420▲, 546▼
	Summary: Genes upregulated by crocidolite exposure could broadly be classified as cell signaling genes, transcription factors, growth factor-related genes, and genes regulating extracellular matrix homeostasis and lung remodeling. Downregulated genes were not discussed.			
(3)	Crocidolite	6 μg/cm^2	A549	24 h: 492▲, 27▼
	Summary: Crocidolite-exposed cells possessed a transcriptome consisting of genes associated mainly with transcription (encoding extracellular growth factors/cytokines and intracellular regulators/mediators of transcription). Additional global analysis demonstrated a network of pathways linked to both apoptosis and cell survival, and allowed for the identification of novel crocidolite-related genes.			
(4)	Crocidolite	1-2 μg/cm^2	A549, MeT5A, Beas-2B	N/A
	Summary: Expression results were examined using gene ontology analysis, cluster analysis, and canonical correlation analysis in order to generate crocidolite asbestos-induced global gene expression profiles in malignant (A549) and non-malignant, immortalized (Met5A, Beas-2B) cells. Doing so allowed for the identification of some novel crocidolite-related genes and biological processes, and distinguished chromosomal regions containing genes which may contribute to shared asbestos-induced responses by these cell lines.			

Note. References: (1) Belitskaya-Levy et al. (2007), (2) Ramos-Nino et al. (2003), (3) Hevel et al. (2008), (4) Nymark et al. (2007). In Results column, numbers represent the total number of genes significantly upregulated (▲) or downregulated (▼) versus controls (>2-fold for Refs. 1 and 3 and >1.5-fold for Ref. 2) at specified time points. N/A, not applicable.

bronchial epithelial (Beas-2B) cells, and SV40-immortalized pleural mesothelial (MET5A) cells (Nymark et al., 2007) to asbestos. Transcript profiling has also been used to identify differentially regulated pathways in lung and tumor tissues of asbestos-exposed and nonexposed lung cancer patients (Ruosaari et al., 2008), as well as to examine gene changes in human pulmonary artery endothelial cells (HPAEC) exposed to ultrafine particles (Karoly et al., 2007). A summary of results from several of these *in vitro* studies using asbestos can be found in Table 3. Therefore, implementing gene profiling as a means of predicting the pathogenicity of several insoluble minerals to human mesothelial (LP9/TERT-1) cells seemed reasonable.

PCA is one of two types of higher order statistical analysis methods that can be employed to extract biologically relevant gene expression features from substantial microarray data sets, with the other being independent component analysis (ICA) (Kong et al., 2008). PCA is essentially a multivariate data analysis procedure involving transformation of a number of possibly correlated variables into a smaller number of uncorrelated variables known as principal components (Jolliffe 1986). Numerous examples exist of this statistical method being applied to microarray data as a means of feature extraction, gene clustering, and classification (Iwakawa et al., 2008; Stevens et al., 2008; Worley et al., 2008). PCA of our laboratory's recently published microarray data (Shukla et al., 2008) demonstrated that LP9/TERT-1 human mesothelial cells were uniquely sensitive to crocidolite asbestos exposure compared to IOSE human ovarian epithelial cells. This is not surprising since the IOSE cell type is not implicated in asbestos-induced diseases and was included essentially as a negative control (Committee on Asbestos: Selected Health Effects, 2006; Reid et al., 2009). More importantly, PCA allowed us to order the fiber/particle exposures based on the magnitude of the cellular response. Doing so demonstrated

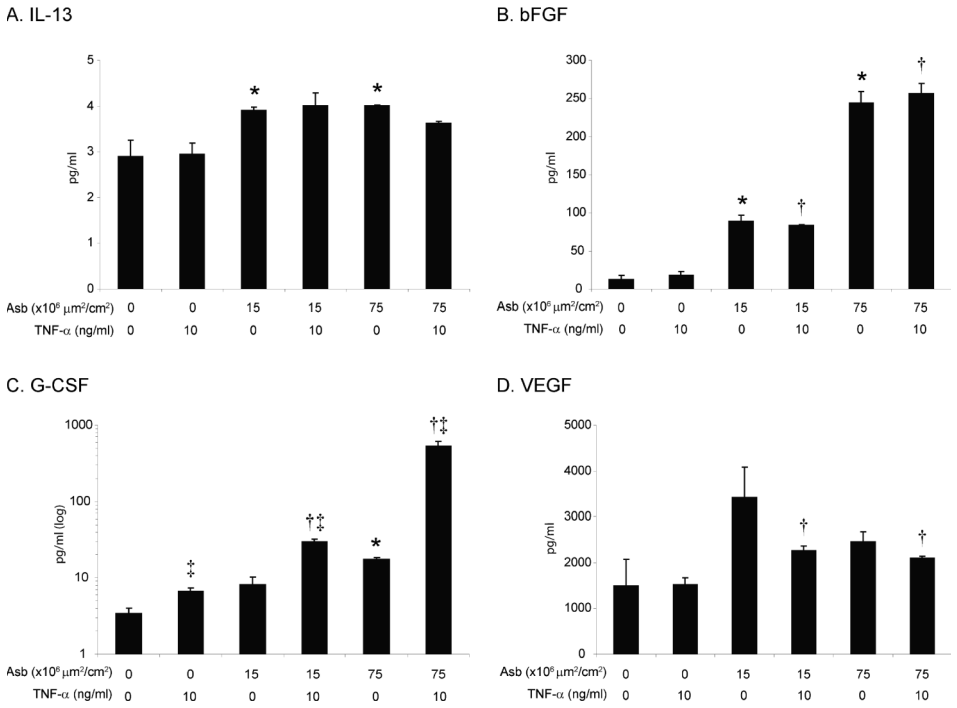

FIGURE 2. Bio-Plex analysis of medium collected from LP9/TERT-1 cells exposed to 15×10^6 or 75×10^6 µm^2/cm^2 crocidolite asbestos for 24 h in the presence or absence of TNF-α (10 ng/ml). Asbestos exposure caused an increase in IL-13 (A), bFGF (B), G-CSF (C), and VEGF (D) at both concentrations tested, although only bFGF and G-CSF exhibited a dose-related response. Concurrent TNF-α priming and asbestos exposure appeared only to affect G-CSF production. Data is represented as the mean ($n = 3$ samples per group) ± SEM. Asterisk indicates significant at $p < .05$ compared to medium control group (–Asb/–TNF-α); †, significant at $p < .05$ compared to medium control group (–Asb/+TNF-α); ‡, significant at $p < .05$ compared to same asbestos concentration (+Asb/–TNF-α).

that crocidolite asbestos induced the largest number of gene changes in a dose-dependent and time-dependent fashion. The ability to order exposures may be of utility in general toxicity assessment. In principle, the observation that exposures lie along a line in Figure 1 may reflect, exclusively, variation in the fraction of cells impacted by the exposure, or variation in the magnitude of the response of individual cells. Since it is clear from microscopy that populations of cells are heterogeneous with respect to exposure to fibers/particles, and since immunocytochemical studies show variation among a population of cells with respect to response, we expect that the former model dominates; that is, the exposures vary with respect to the number of cells impacted.

As stated previously, the pathogenesis of asbestos-associated diseases is most commonly associated with a persistent inflammatory response initiated by ROS, growth factors, and/or various pro-inflammatory factors such as cytokines or chemokines. These secreted factors can subsequently trigger activation of transcription factors and mitogen-activated protein kinases (MAPK), which are linked to early-response genes (Manning et al., 2002). For example, ROS-mediated TNF-α release from macrophages is induced by exposure to asbestos fibers (Donaldson et al., 1992; Simeonova & Luster, 1995). Further, it has been established that TNF-α and IL-1β promote anchorage-independent growth of immortalized human mesothelial cells treated with erionite *in vitro*, and may therefore contribute to the pathogenesis of MM (Wang et al., 2004). *In vivo* models utilizing transgenic mice overexpressing TNF-α in type II alveolar epithelial cells spontaneously develop fibrotic lesions similar to those observed in asbestosis (Miyazaki et al., 1995). Finally, TNF-α receptor knockout mice fail to develop the fibrosis seen in wild-type mice following chrysotile asbestos

exposure (Liu et al., 1998). Hence, TNF-α seemed a logical source to prime LP9/TERT-1 cells to crocidolite asbestos exposure for Bio-Plex analysis.

Results of these studies demonstrated that asbestos caused an increase in IL-13, bFGF, G-CSF, and VEGF under various exposure conditions. Only bFGF and G-CSF responded in a dose-dependent manner and only G-CSF responded in a TNF-α-dependent manner. Specifically, both the 15×10^6 and 75×10^6 $\mu m^2/cm^2$ dose of crocidolite asbestos caused a significant increase in IL-13 production (Figure 2A), and these responses appeared independent of TNF-α stimulation. IL-13 is a cytokine that serves as one of the major effector molecules at sites of Th2 inflammation and lung remodeling (Elias et al., 2003). IL-13 is believed to play a central role in negatively regulating antitumor immunity and enhancing tumor growth (Terabe et al., 2004). Also, IL-13 in the mouse lung activates extracellular signal-regulated kinase 1/2 (ERK1/2), which contributes to IL-13-induced inflammation and remodeling and serves as an integral component both in IL-13 stimulation of multiple chemokines and proteases, and in the inhibition of specific antiproteases (Lee et al., 2006). When considering MM specifically, it has been shown that IL-13 and TNF-α regulate the expression of vascular cell adhesion molecule-1 (VCAM-1) in MM cells (Ruco et al., 1996). Further, significant elevations in IL-13 were observed in wild-type mice exposed to chrysotile asbestos for 3 d, a response that was attenuated in protein kinase C (PKC)-δ knockouts (Shukla et al., 2007).

LP9/TERT-1 production of bFGF and G-CSF occurred in a dose-dependent manner following crocidolite asbestos exposure, although coexposure to TNF-α affected G-CSF production only (Figure 2B and 2C). bFGF, a member of the fibroblast growth factor family of proteins, binds heparan sulfate proteoglycans and is commonly found in malignant tumors of mesodermal and neuroectodermal origin (Muramatsu & Muramatsu, 2008). It is released as a result of tissue injury, inflammation, or tumorigenesis, where it is mitogenic to vascular endothelial cells and works synergistically with VEGF to induce tumor angiogenesis (Bremnes et al., 2006). Previous work has shown that bFGF and heparanase are coexpressed in MM, thereby allowing heparanase to release bFGF from the extracellular matrix to support angiogenesis within the solid tumor (Davidson et al., 2004, 2006). Additionally, epidemiological studies of human subjects heavily exposed to asbestos have demonstrated that these individuals possess higher levels of hepatocyte growth factor (HGF), bFGF, and VEGFβ (Amati et al., 2008). Like bFGF, G-CSF is considered a glycosaminoglycan-binding cytokine that acts by stimulating granulocyte differentiation from progenitor cells (Muramatsu & Muramatsu, 2008). G-CSF is secreted from a number of different cell types and was shown to be expressed in normal human mesothelial cells and human MM cells *in vitro* (Demetri et al., 1989; Schmitter et al., 1992). TNF-α stimulation was required to induce G-CSF expression in normal mesothelial cells (Demetri et al., 1989), which essentially parallels our results in LP9/TERT-1 cells (Figure 2C). Also, numerous patient case reports demonstrate the presence of G-CSF in human peritoneal and pleural MM tumors (Kasuga et al., 2001; Kimura et al., 2005; Nishimura et al., 2006; Ohbayashi et al., 1999; Yoshimoto et al., 2005).

VEGF release by LP9/TERT-1 cells exposed to crocidolite asbestos was more variable than the other proteins examined (Figure 2D). Exposure to 15×10^6 or 75×10^6 $\mu m^2/cm^2$ crocidolite asbestos caused an increase in VEGF, although these changes were not statistically significant ($p < .05$). However, LP9/TERT-1 cells co-treated with asbestos (15×10^6 or 75×10^6 $\mu m^2/cm^2$) and TNF-α were significantly ($p < .05$) different from cells treated with TNF-α alone. VEGF comprises a subfamily of platelet-derived growth factors and is a potent inducer of angiogenesis. It is secreted by multiple cells types including malignant tumor cells, endothelial cells, macrophages, and stromal cells, and targets its mitogenic effects primarily to vascular endothelial cells (Bremnes et al., 2006). We chose to examine this growth factor given recent preclinical and clinical

evidence suggesting angiogenesis is intimately linked to the pathogenesis of MM. Specifically, investigators have shown elevated levels of VEGF in the serum of MM patients (Kumar-Singh et al., 1999; Linder et al., 1998), as well as increased expression in epithelioid type MMs compared to biphasic or sarcomatoid types (Aoe et al., 2006). In similar studies, it has been shown that MM cells also express elevated levels of various VEGF receptors including VEGFR-1 (Flt-1), VEGFR-2 (Flt-2/KDR), and VEGFR-3 (Flt-4). In these cells, VEGF appears to cause receptor-mediated cellular proliferation via a functional autocrine loop, which can be blocked via antibodies directed to either VEGF or its receptors (Masood et al., 2003; Strizzi et al., 2001). Given the integral role played by VEGF in MM progression, it has emerged as a promising therapeutic target, and multiple clinical trials have been conducted or are underway to examine the potential of novel anti-angiogenic agents such as Bevacizumab (VEGF monoclonal antibody) or SU-5416 (VEGFR-2 selective inhibitor) for effective treatment (Catalano et al., 2004; Kindler, 2004; Nowak et al., 2002).

CONCLUSIONS

Microarray (with associated PCA) and Bio-Plex analyses were valuable in determining early molecular responses to crocidolite asbestos. Details pertaining to specific gene or cytokine changes identified by these *in vitro* methods may directly contribute to understanding the etiology of fiber/particle-induced diseases and may aid in the identification of therapeutic targets. Concurrently, the number of changes in gene expression or the "profiles" of secreted proteins may serve as vital metrics for determining the potential pathogenicity of various mineral types, a hypothesis we are testing in ongoing studies. In studies described here, crocidolite asbestos in comparison to other particles caused the largest number of gene changes and cytokines released from cells, and should therefore be considered the most pathogenic fiber/particle tested. This was supported by studies demonstrating that crocidolite asbestos was also the most cytotoxic agent, as determined by trypan blue exclusion cell viability assays in LP9/TERT-1 and IOSE cells (Shukla et al., 2008). It is interesting to note that the asbestos-induced changes seen in IL-13, bFGF, G-CSF, and VEGF protein levels (via Bio-Plex) were not necessarily associated with concomitant changes in transcript levels as determined via microarray. In fact, of these four cytokines/growth factors, only the expression of VEGF had a change >1.5-fold. Specifically, exposure to 15×10^6 $\mu m^2/cm^2$ crocidolite asbestos for 24 h resulted in a 2.1-fold increase in VEGF expression over the medium control group. This lack of correspondence between protein and transcript levels may represent either an alteration in release of these cytokines/growth factors from the cells or some other posttranslational modification, or may indicate posttranscriptional rather than transcriptional regulation.

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